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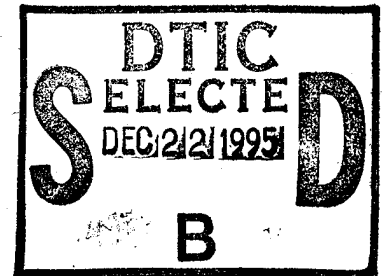
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Growth in the Mammary Gland

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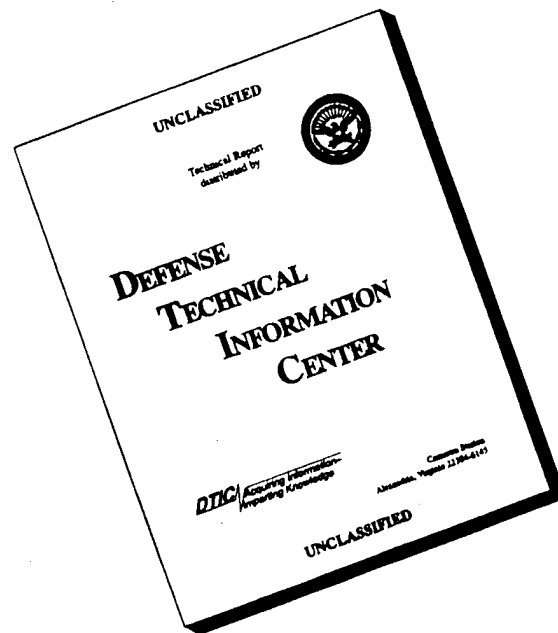
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5. INTRODUCTION

Nature of problem/Background of previous work.

The mammary gland develops from an epithelial out pocketing of the ventral ectoderm at 11dpc in the mouse embryo [1-4], in response to an initial inductive signal from the underlying mesenchyme [5]. In the female mouse embryo, there is little change in the primary bud over the next four days. However, in the male, mesenchyme surrounding the epithelium condenses from day 14 and this is followed by a rapid necrotic degeneration of the epithelial rudiment. Tissue recombination experiments have convincingly demonstrated that this process is dependent upon testosterone [6], and correlates with the acquisition of testosterone receptors by the mammary mesenchyme [7], which occurs in response to epithelial derived signals [8], and the initial production of testosterone by the embryonic testes. In addition to testosterone responsiveness, the epithelium also induces estrogen responsiveness but, at this stage of development, there appears to be no *in vivo* role for estrogen [1]. Thus, by the end of this resting period, which is characterized by the appearance of the mammary bud (16.0 dpc), the female mammary gland is poised for further development whereas the male gland is destroyed.

From 16.0dpc to 2dpp, the mammary epithelium extends as the primary mammary sprout into the mesenchyme reaching the fat pad precursor where epithelial branching is initiated. The trigger for elongation of the mammary epithelium is not known, however outgrowth follows the resumption of proliferative activity in the epithelium. The trigger for epithelial branching clearly resides in the mesenchyme of the fat pad precursor. Epithelial morphogenesis, which forms slim epithelial ducts with secondary lateral buds, is specific to the fat pad mesenchyme [9]. Growth into other sources of mesenchymal tissue *in vitro*, e.g. salivary mesenchyme, produces epithelial outgrowths typical of the organ from which the donor mesenchyme was removed. [10]. Thus, shortly after birth in the mouse, and in other mammals [2], the female mammary gland consists of a primary duct connecting with a rudimentary branched epithelium within the presumptive fat pad.

From the period after birth to approximately 4 weeks pp there is very limited growth of the branching epithelium of the mouse mammary gland. However, at 4 to 6 weeks, coupled with the acquisition of sexual maturity, there is a period of extensive cell growth in which the epithelial ducts elongate and branch, extending throughout the fat pad. [11,12] Renewed growth correlates with the reappearance of end buds, a monolayer of unspecialized epithelium at the ends of the ducts. The end buds are thought to contain stem cells which generate differentiated ductal epithelium and myoepithelial cells of the gland. Thus, post natal branching morphogenesis is regulated largely at the termini of the ducts, by controlling the proliferative activity of the end buds. End bud activity is in turn dependent upon ovarian hormones, as ovariectomy results in a rapid loss of end buds and cessation of growth [11,12].

After reaching sexual maturity (6 to 8 weeks pp), further ductal development stops until pregnancy is established. At this time, a second extensive period of ductal growth and branching occurs to fill all the remaining interductal space in the fat pad. During the later phase of pregnancy there is an accompanying development of lobuloalveolar epithelium. Along with the morphogenetic changes in the gland during pregnancy, there is a progressive development of secretory epithelium, such that by birth, a fully functional lactogenic epithelium is established. Interestingly, cytodifferentiation of secretory epithelium will occur *in vitro* in the absence of morphogenesis, indicating that the two processes are not mutually dependent [13]. Finally, on cessation of suckling, there is a massive involution of the mammary gland due to a widespread destruction of epithelial tissue and the cycle of branching morphogenesis is repeated at the next round of pregnancy.

The mammary gland is unusual, with respect to most organs, in that most of its growth occurs in the adult, and that there are cyclical periods of growth and regression. The control of these processes has been extensively studied and compelling evidence exists for complex regulation mediated by systemic hormonal signals, and locally acting peptide growth factors (for review see [11,12]).

The initial observation that ovariectomy leads to a cessation of end bud growth implicated hormones in the control of mammary development. There is an absolute requirement for estrogen for proper development of epithelial branching. Maximal growth also appears to require growth hormone or prolactin [11,12]. However, whether these hormones act directly, or sensitize the epithelium to the action of other factors is not clear. Lobuloalveolar growth requires, in addition to the above, progesterone which accumulates later in pregnancy. Finally, the onset of lactation correlates with the increase in prolactin and glucocorticoids and a decrease in progesterone [11,12].

Evidence for involvement of peptide growth factors in the regulation of mammary development has come from the direct observation of growth factor expression, and implant and transgenic studies which have manipulated growth factors in the mammary gland. Slow release implants of EGF stimulates local growth of end buds in quiescent mammary epithelium [14], whereas implantation into growing mammary glands causes local inhibition of ductal growth, and a down regulation of EGF receptors [15]. Thus, EGF may have a dual specificity depending upon the particular stage of development. TGF- β 1 implants also suppress ductal growth [16] acting specifically on the end buds to inhibit DNA synthesis [17] whereas TGF α stimulates alveolar and ductal growth [18-21].

Additional evidence for peptide factors in growth regulation has come from the analysis of mammary tumors in which growth controls have been uncoupled following expression of genes not normally active in the mammary gland. A number of loci, have been shown to undergo MMTV mediated insertional activation in mouse mammary tumors (for review see [22]). Four of these encode secreted peptide factors, *Wnt-1* [23] and *Wnt-3* [24] members of the *Wnt*-gene family, and *FGF-3* [25] and *FGF-4* [26], members of the fibroblast growth factor family. Additional evidence suggests that *Wnt* and *FGF* genes may cooperate in tumor formation as frequently *Wnt-1* and *FGF-3* are co-activated in the same mammary carcinomas [27].

The oncogenic role of *Wnt-1* has been demonstrated by *in vitro* and *in vivo* studies. Transfection of the *Wnt-1* gene into C57MG cells, a primary mammary epithelial cell line leads to morphological transformation [28,29]. However, these cells do not grow in soft agar or form tumors in syngeneic hosts. In contrast RAC311C cells are rendered morphologically transformed and tumorigenic when transfected with *Wnt-1* [30]. Formal proof of the transforming roles of *Wnt-1* has come from transgenic studies which lead initially to hyperplasia, in both the male and female mammary gland, and progress to the formation of adenocarcinomas [31]. As was observed in spontaneously occurring tumors, there is also a synergistic affect of *FGF-3* on *Wnt-1* transformation in the transgenic model [32].

In addition to *Wnt-1* and *Wnt-3*, ten additional members of the mouse *Wnt*-gene family have been identified. Human *Wnt-2* was isolate serendipitously in a search for the cystic fibrosis gene [33,34]. Like *Wnt-1* and *Wnt-3*, *Wnt-2* is implicated in tumorigenesis as it appears to be amplified and highly expressed in some MMTV induced tumors [35]. Amplification appears not to be related to MMTV, but is a novel mechanism which presumably acts in conjunction with MMTV activated genes to transform epithelial cells [35]. *Wnt-3a* was identified on the basis of its close relationship to *Wnt-3* [36], and *Wnt's-4, 5a, 5b, 6, 7a, 7b*, on the basis of a PCR cloning approach [37] which has been successful in identifying *Wnt*-genes in many species, as well as two new mouse members (*Wnt-10* and *11*; A. McMahon, unpublished data).

All *Wnt*-proteins, have several features in common including a putative signal peptide sequence, one conserved glycosylation site and 20 absolutely conserved cysteine residues. Typically *Wnt* proteins are 38 to 45kd. Although only *Wnt-1* and *Wnt-2* have been studied, and these analyses have been restricted to cell culture systems, both genes appear to encode poorly secreted glycoproteins with strong affinity for cell surface and/or extracellular matrix [29,38-44]. Thus, it is likely that they are involved in short-range signaling. Functionally analyses of several members indicates these important regulatory roles in invertebrate and vertebrate development reviewed in [45,46].

The observation that *Wnt* expression leads to morphological transformation of mammary epithelial cells *in vitro* and hyperplastic growth *in vivo* indicates that mammary epithelium is responsive to *Wnt* gene products. If, *Wnt*-proteins act as signals (a conclusion greatly strengthened by studies on the *Drosophila* *Wnt*-1 orthologue *wingless*, [46]), then by analogy with other families of peptide signals, it would seem likely that the responsiveness of mammary epithelium reflects the expression of functional *Wnt*-receptors.

Recent evidence demonstrates that unlike, *Wnt-1* and *Wnt-3*, six family members are expressed, and developmentally regulated, during normal adult mammary gland development [47]. Thus, the responsiveness to ectopic expression of *Wnt-1* or *Wnt-3* presumably reflects some modulation of *Wnt*-signaling pathways which normally respond to endogenously expressed *Wnt*-factors. For example, if *Wnts* normally stimulate cell growth, ectopic expression of *Wnt-1* or *Wnt-3* may lead to hyperstimulation of a proliferative *Wnt*-signaling pathway. Conversely, if endogenously expressed *Wnts* suppress proliferative activity, ectopic *Wnt-1* or *Wnt-3* expression may block *Wnt*-mediated growth suppression, possibly by interfering with receptor function.

The situation is likely to be complex on the basis of our studies of *Wnt*-transcription in the adult mammary gland [47b]. *Wnt-2* expression is very weak and confined to virgin or nonpregnant mice [47b]. Thus although *Wnt-2* causes C57MG cell transformation, its expression does not correlate with proliferative activity. Quite the opposite, it is limited to the quiescent state. *Wnt-5a* and *Wnt-7b* are also expressed at low levels in virgin mice [47]. However, expression extends into mid but not late pregnancy showing decreasing levels of expression despite the large increase in mammary epithelium. In contrast, *Wnt-5b* and *Wnt-6* are expressed at low levels prior to pregnancy and increase considerably to midpregnancy, declining by parturition [47]. Thus, these two members show a better correlation with epithelial expansion. Finally *Wnt-4* expression is uniform from in the virgin gland until late in pregnancy when it rapidly declines [47].

Transformation assays on C57MG cells indicate that several *Wnt*-members which are normally expressed in the mammary gland are transforming in this assay [48]. *Wnt-2*, -5b and -7b are moderately transforming, weaker than *Wnt-1*, 3a and 7a, whereas *Wnt-4*, 5a and 6 are non transforming. *Wnt-4* and *Wnt-5a* are normally expressed by C57MG cells, thus elevation of endogenous expression several fold does not lead to transformation. These results suggest that hyperplasia *in vivo* may result from inappropriate activation of *Wnt-2*, *Wnt-5b* and/or *Wnt-7b* signaling pathways. On the basis of crude expression studies, the *Wnt-5b* pathway may be the best candidate.

In summary, the data clearly support a model in which normal mammary, epithelial growth is regulated by one or more *Wnt*-genes. They demonstrate that uncoupling of these regulatory pathways leads to hyperplasia [31,49] and adenocarcinomas *in vivo* [31]. However, without a better understanding of the normal spatial expression of *Wnt*-proteins and their putative receptors, and the transforming activity of the family as a whole *in vivo*, we are not in a position to grasp the full significance of their functions in the normal and transformed mammary tissue, nor the relevance that this family may have to human breast cancer.

Purpose of present work/Methods of approach

As discussed above it is now over ten years since Nusse and Varmus identified a locus in the mouse associated with the generation of mammary tumors. It is now clear that the associated gene, *Wnt-1*, is one member of a large family of putative signaling molecules which normally regulate embryonic development. Several members have now been implicated in epithelial cell transformation in the mammary gland from the analysis of spontaneously occurring mouse tumors (*Wnt-1*, *Wnt-3*, *Wnt-3a*), transgenic experiments (*Wnt-1*) and *in vitro* studies (*Wnt-1*, 2, 3, 3a, 5b, 7a, 7b). Thus, it would appear that hyperplasia, and eventual adenocarcinoma formation, in the mouse mammary gland result when normal growth regulatory pathways which are presumably controlled by *Wnt*-proteins, are perturbed by deregulated expression of certain *Wnt*-family members.

Since understanding growth control in the mammary gland is essential for designing strategies which will treat mammary tumors. Further, potential growth regulators are likely mediators of mammary transformation, as exemplified by studies on *Wnt*-genes in the mouse, and should thus be examined for contributory roles in human mammary cancer. This proposal set out to examine the normal and oncogenic roles of *Wnt* protein in the mammary gland of the mouse and human, and to dissect the *Wnt*-regulatory pathways at the receptor level. Specifically we proposed to address the issue of whether *Wnt*-genes may be involved in human cancers by directly examining expression in mammary tumors using Northern blot analysis. We propose to use transgenic mice to examine the relationship between normal *Wnt*-gene expression and mammary transformation. As *Wnt*-signaling is most likely a conventional receptor mediated process, ectopic expression of specific *Wnt*-signals presumably exert their effects through, one or more, receptor pathways coupled to endogenously expressed *Wnt*-proteins. If so, we should be able to identify a likely candidate pathway by assaying the transforming potential of endogenously expressed *Wnt*-proteins when their normal regulation is uncoupled, either by ectopic expression or gene ablation. Moreover, characterizing the normal expression of *Wnt*-genes and their products in relation to the developing mammary gland, may provide strong suggestive evidence as to what growth regulatory pathways may be responsive to *Wnt*-signals. Finally we propose several approaches toward identifying *Wnt*-receptors which will be an essential step in fully defining *Wnt*-signaling pathways, and their regulatory function in the mammary gland. Thus, the proposed studies are directly relevant to the issue of the genetic alterations involved in the origin and progression of cancer and the changes in cellular and molecular function which may account for the development and progression of breast cancer.

In summary we proposed five specific goals

- 1) To determine, using transgenic mice, which if any of the *Wnt*-members normally expressed in the mammary gland are oncogenic when ectopically expressed using an MMTV enhancer construct.
- 2) To determine the relevance, if any, of *Wnt-5b* in normal gland development by studying mice homozygous for a likely null mutation in the *Wnt-5b* gene.
- 3) To determine the normal temporal and spatial expression of *Wnt* genes, and their protein products, during embryonic and adult mammary gland development.
- 4) To use various schemes to attempt to identify other proteins, particularly candidate receptors, which interact with *Wnt*-proteins.
- 5) To isolate sequences encoding all of the yet unidentified human *Wnt*-genes provide clinicians with a broad array of *Wnt*-probes which may be important in the analysis of human mammary carcinomas.

6. BODY

Over the past year we have made considerable progress in certain areas and these have suggested some exciting new avenues to follow up. Thus, the research goals as they appeared in the original proposal have been substantially modified to take into account our new findings. These substantial changes will be discussed in the next section but we would like to stress at this time that following up the new results require that we drop some of the original goals.

1) Transgenic analysis of Wnt-mediated oncogenesis

Wnt-1 and *Wnt-3* were both identified on the basis of their role in MMTV derived mammary tumors. However, it seems likely that these two genes stimulate hyperplastic growth, a first step towards the generation of adenocarcinomas, by deregulated Wnt-mediated growth regulation in the mammary gland. One strong possibility is that one or more of the *Wnt*-members normally expressed in the mammary gland are growth stimulatory, and it is through this pathway that *Wnt-1* and *Wnt-3* act. If this is the case, deregulated expression of these members would be predicted to act like *Wnt-1* and *Wnt-3* causing hyperplasia initially, and tumors with time. Thus, a transgenic approach, utilizing endogenously expressed *Wnts*, may provide an insight into the regulatory pathway through which *Wnt-1* and *Wnt-3* act.

We proposed to test for the transforming potential of *Wnts-2, 4, 5b, 6, 7b*; the six members which we had previously shown to be expressed in the mouse mammary gland, by ectopic expression utilizing an MMTV-LTR, as previously reported for *Wnt-1* [31].

We have not pursued these studies in the current funding period preferring to change the focus towards an understanding of earlier development and the relevance of *Wnt-6* and the newly identified *Wnt-10* in embryonic mammary gland development (see later).

2) *Wnt-5b* mutant analysis

One approach towards examining the role of Wnt members during mammary development is to generate specific mutants by gene targeting in ES cells. To date we have mutated three of the six mammary expressed genes (*Wnt-4, Wnt-5b, Wnt-7b*), but due to embryonic requirements, only *Wnt-5b* homozygous mice are viable. *Wnt-5b* was mutated by insertion of a PGK-neo cassette into the fourth exon. Insertion produces a short deletion and places PGK-neo into the normal *Wnt-5b* open reading frame. Thus the mutated allele is predicted to produce a carboxyl truncated protein. Our experience with similar truncations suggested that this would generate a null allele [74]. *Wnt-5b* is moderately transforming in the C57MG assay, suggesting that *Wnt-5b* may play a role in epithelial growth stimulation. Moreover, expression of *Wnt-5b* increased dramatically through the first half of pregnancy, a period characterized by the reemergence of mitotic activity in the end buds and reinitiation of branching morphogenesis.

At the time of writing the proposal we had weanlings (3-4 weeks pp) which were homozygous for *Wnt-5b* the disrupted allele. We proposed to determine whether loss of *Wnt-5b* activity leads to a disruption of growth regulation in the mammary gland, by examining the histology and function of the mammary gland in mutant mice. It is now clear that both males and females are fully viable. Moreover, homozygous females, are perfectly able to suckle their offspring and therefore it is likely that mammary development is quite normal.

There are several possible explanations for this result. The simplest would clearly be that *Wnt-5b* by itself does not play an essential role in mouse mammary gland development. This may be true, however, this result presupposes that the targeted allele is indeed a null allele. Our Southern blot analysis of the targeting event indicates that the expected recombination event occurred. However, there is now some question as to whether the allele we have generated is indeed a null allele. Unpublished work from the laboratory of Dr Eric Wieschaus indicates that a similar allele of *wingless*, one in which the carboxyl one quarter of the protein is missing is in fact a hypomorph, a surprising result given that the mutation removes ten absolutely conserved cysteine residues. Thus, to be absolutely certain that *Wnt-5b* plays no role, we must generate a second unambiguous null allele. We can generate this allele by simply deleting exon 4, with little

modification to the original targeting strategy. Moreover, as the gene targeting frequency is reasonable (approximately 1 in 80 neo/TK resistant clones), this approach should not be too difficult.

3) Wnt expression in the mammary gland

Although we have demonstrated that several *Wnt* genes are expressed during mammary development, we do not know in which cell types, nor the spatial details. This is of critical importance. Growth and branching morphogenesis is primarily regulated at the end buds. Thus, any growth stimulatory or growth repressive action of a Wnt member is likely to act on this aspect of the epithelial network. As the available evidence suggests that Wnts are short range factors, we would therefore anticipate that some Wnt members will be locally distributed either in the stroma surrounding the end buds, or perhaps in the end bud themselves, and their expression would be predicted to change dramatically with development. Clearly, it is essential for our understanding of their normal regulatory roles that we determine their expression patterns. We proposed to examine this problem by both in situ hybridization and the use of antibodies raised in the laboratory against many of the Wnt-proteins. Further, no one has yet addressed the possibility that Wnt proteins may play a significant role in the early interactions which induce and elaborate the embryonic mammary bud. We proposed to use in situ hybridization to determine whether Wnt-signaling may be involved.

Our initial approach was to use antibody reagents to attempt to immunostain sections and wholemounts of mouse embryos. We chose to examine embryos in the first instance because we have documented sites of Wnt-expression in different regions of the mouse embryo in some detail in our published work. Moreover, we know that several of these sites produce active Wnt-protein as Wnt-mutants are defective in the development of these tissues. We have tested antisera directed against Wnt-1, 3a, 4a, 5a, 5b, 7b but none of these give a reliable, signal despite a number of different fixation conditions (paraformaldehyde, methanol/DMSO, TCA). Thus, we discontinued this approach. It is worth pointing out that this is a considerable problem in the field in general. To date NO ONE has visualized the normal expression of a Wnt-protein, in situ, in any vertebrate embryo. At this time we have not attempted the alternative strategy on adult tissues, that is the RNA in situ hybridization approach, but we have used this approach on whole mounts of mouse embryos, and obtained some interesting results. We have identified two Wnt-genes that are expressed in association with the earliest stages of mouse mammary gland development. These are *Wnt-6* and *Wnt-10*.

Wnt-6 was identified in my laboratory a number of years ago [37]. At the time when the mammary epithelium first thickens, at the initiation of mammary gland development, *Wnt-6* is strongly expressed in the epithelium. Expression of *Wnt-6* is not restricted to the mammary bud epithelium but is present in all ectoderm, suggesting that it may play a widespread role in epithelial signaling. In contrast *Wnt-10* is expressed specifically at two sites of mesenchymal-epithelial interactions, the tooth and mammary buds (Figure 1 in Appendix). Thus, *Wnt-10*, which is a novel mouse family member identified in our PCR-based screens, is a prime candidate for a *Wnt* which might actually induce mammary gland development. In view of the interesting early expression of these members, we will focus our subsequent analysis on determining in detail the expression and properties of *Wnt-6* and *Wnt-10* (see Conclusions)

4) Wnt receptors

A major roadblock to our understanding of the regulatory function of Wnt-proteins, in embryonic development, as well as in the mammary gland, is our limited knowledge of the signaling pathways. In the absence of compelling data to the contrary the simplest hypothesis to explain all studies on Wnt-factors is that they encode signals which interact through some receptor mediated pathway. The identification of the *Drosophila* segment polarity gene *wingless* as the Wnt-1 orthologue raised the possibility that *Drosophila* genetics would identify a receptor. Indeed at least two genes, *armadillo* and *disheveled*, have the requisite characteristics, that is mutants in these genes have a wingless phenotype which acts cell autonomously [46]. However, *armadillo* encodes a plakaglobin/ β -catenin counterpart, and is thus implicated in binding to functional complexes or adhesive proteins whereas *disheveled* encodes a cytoplasmic protein of

unknown function [46]. More recently, it has been argued on the basis of genetic interactions that Notch, a large multi-functional transmembrane protein might encode a wingless receptor, but a direction action has not been demonstrated.

Wnt-proteins are poorly secreted and interact with extracellular matrix and/or the cell surface. Thus far no group has succeeded in purifying large quantities of secreted protein a necessary step before attempting to affinity purify a putative receptor. The problem clearly requires novel strategies. The importance of this issue to the cancer problem warrants that we attempt some more speculative approaches.

A) SURAMIN AFFINITY CHROMATOGRAPHY

In the first approach we thoroughly characterized the properties of several Wnt-proteins expressed by cos cells, demonstrating the differential effects of poly anionic compounds such as suramin and various analogues thereof, and heparin on the secretion of Wnt-proteins. We made good progress with this work and it is now in press [**L. Burrus and A. P. McMahon (1995) Biochemical analysis of murine Wnt-proteins reveals both shared and distinct properties. Experimental Cell Research, in press**]. We then attempted to purify active soluble Wnt-ligands. The strategy was to express the proteins at high levels in cos cells, and to attempt to purify the ligands from either the medium or supernatant by affinity chromatography on heparin or suramin columns. Our experience to date is sobering, and has lead us to discontinue this approach. Firstly, it is difficult to express Wnt-proteins to high levels in the cos cells, and obtain efficient secretion. Thus, the amount of protein present in the medium is very small and requires a massive scale up to isolate even nanogram quantities. As for the majority of protein in the cell, this is likely to be insoluble in the endoplasmic reticulum, and may represent mis-folded protein. The finding that wingless is not secreted efficiently in *porcupine* mutant embryos suggests that there may be specific accessory proteins required for effective Wnt-secretion, a significant hurdle to overcome. Of the protein we obtained from the columns, we were unable to enrich for Wnt-protein on the suramin column, but obtained some enrichment on heparin. However, it does not seem that purifying the intracellular protein is a sensible strategy.

B) THE TWO HYBRID SYSTEM

A second approach we suggested is to use the yeast two hybrid system [50, 51], with a Wnt-bait to trawl for a Wnt- receptor. In this system, which has now been used to address multiple types of protein-protein interactions, a GAL4 DNA binding domain is fused to the Wnt-ligand (in our case Wnt-3a was used in the initial test) and yeast transfected with a cDNA library from an appropriate source (in our case a 9.5 dpc embryo) in which inserts are fused to a GAL4 transcriptional activation domain. If the Wnt protein can bind to its receptor under these highly artificial conditions (in the nucleus of a yeast cell), then DNA binding and activation domains are brought together allowing transcription of reporter genes (in our case for histidine synthesis and LacZ expression) to be initiated and potentially selected for. These experiments initiated in my laboratory by Dr Marty Shea, are now part of a collaboration between Dr Shea, Dr Allan Bradley and myself, following the move of Dr Shea to Dr Bradley's laboratory.

Initial results are very exciting. Out of a randomly primed whole 9.5 dpc mouse embryo library, the Wnt-3a bait specifically selected a mouse Notch homologue, Notch 3, as a target. In view of the *Drosophila* data it would appear that Notch members are indeed good candidates for Wnt-receptors. This is the first demonstration of a direct biochemical interaction between these proteins and is likely to be of considerable interest. This unexpected result has lead us to revise our experimental goals, and initiate new lines of investigation to determine whether this initial result is indeed significant (see Conclusions).

5) Human Wnt-clones

The demonstration that deregulated expression of Wnt-genes contributes to adenocarcinomas of the mouse mammary gland suggests that Wnt-family members may play a role in human breast cancer. This problem has been only superficially addressed. Using PCR primers we isolated exonic sequence for human *Wnt-3a*, *4*, *5a*, *7a*, *7b*, and together with available probes for *Wnt-2*

and -3, Wnt-transcription was examined in normal, benign and malignant breast tissue [52]. Some evidence for increased expression of *Wnt-2* and -4 in fibroadenomas and *Wnt-7b* in malignant tumor was obtained. To systematically address this problem will require the identification of probes for all human Wnt clones. As this is easily achieved, and these probes will be valuable to clinicians, we propose to complete this task by PCR cloning the remaining family members and making these available.

This was a relatively minor goal in our original proposal. Since submission of the grant it has come to our attention that several groups are systematically exploring the human *Wnt*-family. We have entered into a collaboration with one of these, Dr Tom Strachan, in Newcastle, England, and are sharing our mouse expression data with him. Through our initial efforts and Dr Strachan's more recent work, there are human counterparts for almost all the mouse Wnt-genes, including *Wnt-10*, which given our findings in the mice may be of considerable interest to follow up in the human. Dr Strachan has been made aware of our results and will initiate his own efforts to study the human side of *Wnt* genes. We will focus at this time on mouse studies.

7. CONCLUSIONS

There are several implications of the studies thus far.

1) Transgenic analysis of Wnt-mediated oncogenesis

Clearly the transgenic studies as originally proposed are well within the scope of this laboratories expertise. However, in the light of our discoveries in other areas over the last year, we believe that our resources are best focused on other aspects of the proposal which are likely to lead to more original findings. For example, the role of the newly identified family member, *Wnt-10*, as a possible initiator of mammary development. Thus, we will modify our transgenic studies to two experiments. In the first we will attempt to determine whether *Wnt-10* is oncogenic, using the original strategy. In the second we will test whether *Wnt-10* can induce ectopic mammary gland development by expressing it broadly in the ectoderm using a K14 keratin promoter. This promoter is expressed at the appropriate stage, 11.5 dpc, and has been successful in ectopically expressing a number of genes in the skin [53].

2) *Wnt-5b* mutant analysis

There is no obvious phenotype in mice homozygous for an insertion in the *Wnt-5b* locus. There is some reason to doubt whether the mutation really generates a null allele. Unfortunately there is no simple way to assess whether the targeted allele has partial activity. Thus, we will retarget the *Wnt-5b* locus, deleting exon 4, generating an unambiguous null allele.

3) *Wnt* expression in the mammary gland

We have determined that our antibodies are not likely to be useful for direct immunolocalization of Wnt-proteins and we will not pursue this aspect further. We have identified two Wnt members, *Wnt-6* and *Wnt-10*, which are expressed in the early mammary epithelium at the initiation of mammary gland development. We will concentrate our efforts on addressing the early aspects of mammary gland induction and growth as it occurs during embryonic life. It is likely that at least some of the signals which operate in the embryo are likely to be important in the adult as well as new cycles of growth and differentiation take place. Moreover, studies on the embryonic regulation of mammary gland development have clearly lagged behind the research effort on the adult structure. Thus, this shift in emphasis, which plays to the strength of this laboratory seems a timely one.

There are several straight forward issues to be addressed. For example we need to perform a detailed in situ hybridization analysis throughout embryonic development to determine the precise details of *Wnt-6* and *-10* expression. Further, we only have a small PCR fragment encompassing a part of *Wnt-10*. Thus, it will be essential for functional studies such as those described above to isolate cDNA clones encompassing the complete open reading frame, by screening embryonic cDNA libraries. Finally, we will initiate gene targeting experiments to determine the function of both *Wnt-6* and *Wnt-10*. To this end we have isolated and are mapping genomic clones for both genes.

4) Wnt receptors

The problem of how Wnt-proteins signal, most specifically their receptors, is important not only to our understanding of the many important roles that Wnt-proteins play in the regulation of invertebrate and vertebrate development, but also to their oncogenic actions in the mammary gland. We have characterized the biochemistry of Wnt-proteins and this work is now in press. However, we consider that it is unlikely on the basis of our preliminary work on Wnt protein purification that we will be able to purify sufficient ligand to attempt to clone or characterize a Wnt-receptor by conventional biochemistry. Thus, we will not pursue this work approach any further. However, the yeast screens have turned up a plausible candidate, a Notch-3, and we will vigorously pursue this lead. Clearly there are many things that need to be done. For example, we should address whether the interaction between Wnt-3a and the EGF repeats of Notch-3 identified in the screen are specific, by cloning other EGF repeats into the yeast vector. Moreover, we should determine whether other Wnts can interact with these repeats in Notch-3, or other mouse Notch genes. Clearly, if Notch-3 is a Wnt-3a receptor, it must be expressed in the primitive streak and forebrain where Wnt-3a is functional. Further, it should be possible by expressing an extracellular form of the pertinent EGF domains without the rest of the protein to block Wnt-signaling (dominant negative). Perhaps the simplest approach to this end is to perform

injection studies in *Xenopus laevis*. When *Wnt-3a* RNA is injected into the ventral part of the embryo, it will induce a second axis to form. We can simply test whether co-injection of the dominant negative construct suppresses this activity. Finally, if there is an overwhelming body of evidence in support of a direct Wnt-Notch interaction with a specific set of EGF repeats, we can design a gene targeting approach which will remove this region in the context of the wild type molecule to specifically test the significance of this interaction in vivo.

5) **Human *Wnt*-clones**

We have not pursued the cloning of additional human *Wnts* as this would duplicate efforts in other groups that we have become aware of since submission of this grant. However, we will continue to share unpublished data to facilitate a rapid follow up of potentially interesting areas such as the expression of *Wnt-10*, which may have relevance to human breast cancer, in our collaboration with groups in England.

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9. Appendix

Figure 1. Wholemount in situ hybridization of a *Wnt-10* cDNA probe to the mammary buds of a 14.5 dpc mouse embryo.

Figure 1 - Expression of *Wnt-10* in the embryonic mammary gland

